The Booroola (Fec^B) and Inverdale ($FecX^I$) genes influence ovarian development in early foetal life

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ABSTRACT

The aim of this study was to gain evidence that the Fec^B and $FecX^I$ genes influence ovarian development in foetal life. Foetuses which were homozygous carriers of the Booroola gene (BB), Inverdale gene (II), heterozygous carriers of the Inverdale gene (I+) and non-carriers of both the Booroola (++) and Inverdale genes (++) were recovered at days 40 or 90 of gestation. To eliminate the effects of litter size in the Booroola study, equal numbers of BB and ++ embryos were transferred to recipient ewes. Subsequently no gene-specific differences in litter size were noted at the equivalent of days 40 and 90 of gestation. From these studies the Fec^B gene was shown to affect body weight, the weight of the mesonephros, and development of germ cells. In essence, at day 40 the mean body weight and mean weight of the mesonephros were lighter in the BB foetuses compared to the ++ animals ($P<0.05$). Moreover the development of the BB ovaries appeared to be retarded relative to that of the ++ ovaries with respect to mitosis and meiosis of germ cells and also the timing of germ cell atresia. In the Inverdale foetuses, which were recovered after natural matings, differences were observed at the level of the ovary but none were noted with respect to body weight, or weight of mesonephros. At day 40 of gestation the ovaries of the putative II foetuses were significantly lighter ($P<0.05$) than those of the I+ or control animals. Moreover at day 90 the ++ and putative II ovaries contained significantly fewer ($P<0.05$) germ cells (i.e. ~110,000) compared to those in the authentic or putative I+ animals (i.e. ~240,000). The differences in either the Booroola or Inverdale foetuses were not accompanied by any significant differences in plasma or pituitary concentrations of follicle-stimulating hormone or plasma inhibin. The results are consistent with the notion that the Fec^B and $FecX^I$ genes influence ovarian development in foetal life.

Keywords: Mesonephros, pituitary, ovary, FSH, inhibin, germ cells, oogonia, oocytes, follicles.

INTRODUCTION

Ewes which are homozygous (BB) or heterozygous (B+) carriers of the Fec^B gene or heterozygous (I+) carriers of the $FecX^I$ gene have increased ovulation rates relative to non-carriers of these genes (Davis *et al.*, 1982; Piper and Bindon, 1982; Davis *et al.*, 1991). In contrast, homozygous (II) carriers of the Inverdale gene contain primitive 'streak' gonads and are sterile (Davis *et al.*, 1992).

Several studies on Booroola ewes both before and after puberty have shown gene-specific differences in both pituitary and ovarian function (Bindon *et al.*, 1985; McNatty *et al.*, 1987; Braw-Tal and Gootwine, 1989; Montgomery *et al.*, 1989). At present, there is only limited information on pituitary or ovarian function of Inverdale ewes (Shackell *et al.*, 1993). Nevertheless, the question arises as to whether the Fec^B and $FecX^I$ genes influence gonadal formation or some other aspect of development in foetal life.

The aims of the present study were to examine body weight, the weights of the mesonephros and/or the ovary and the adrenal, together with ovarian germ cell development at days 40 and 90 of gestation. Also examined were the plasma concentrations of inhibin and follicle stimulating hormone (FSH) and the pituitary concentrations of FSH at day 90 of gestation.

METHODS

The experimental procedures reported in this study were carried out in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand after approval was granted by the Animal Ethics Committee of the Wallaceville Animal Research Centre.

All Booroola sheep in this study were half New Zealand Romney, half Merino breed type. Female foetuses from ewes which were homozygous carriers (BB) or non-carriers (++) of the Fec^B gene were recovered from pregnant Booroola ewes following embryo transfer. The BB and ++ donor ewes were superovulated using pregnant mare's serum gonadotrophin (PMSG, Folligon, Intervet, Lane Cove, Australia) and Ovagen (Immuno-Chemical Products Ltd, Auckland, N.Z.). Briefly, 13 BB and 16 ++ Booroola ewes were fitted with intravaginal progesterone devices (CIDR-s type G; AHI Plastic Moulding Co., Hamilton, N.Z.) for 10 days (day of CIDR-s implant = day 0). On day 10 the CIDR-s device was replaced with a second CIDR-s device and the ewes given an i.m. injection of PMSG (300 i.u.) and 8 x 1 ml injections (i.m.) of a 20 ml Ovagen ampoule starting from the time of PMSG injection (a.m.) and thereafter at intervals of 12 h. The second CIDR-s device was removed on the morning of day 13. On the evening of day 13 and on the morning of day 14 the BB ewes

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were mated with BB Booroola rams (n=6) and likewise the ++ ewes were mated with ++ Booroola rams (n=6). Embryo recovery and non-surgical transfer was undertaken 4 days later to oestroussynchronized Romney ewes each of which received three embryos (one ewe received four embryos). Subsequently 18 BB and 10 ++ female foetuses were recovered from the recipient ewes at the equivalent of day 40 of gestation and 14 BB and 14 ++ females at the equivalent of day 90 of gestation.

Female foetuses from Inverdale ewes were recovered from pregnant ewes at days 40 and 90 post mating. Matings of carrier (I) rams with control ewes (++) gave female foetuses all of the heterozygous (I+) genotype at day 40 (n=10) and at day 90 (n=10). Matings of I rams with I+ ewes produced homozygous (II) or heterozygous (I+) foetuses (n=9 II or I+ at day 40 and 15 II or I+ at day 90) while matings of control Romney rams with ++ ewes produced the ++ controls at day 40 (n=10) and at day 90 (n=14).

As each foetus was recovered the body weight, crown-rump length and ovarian weight was recorded. Also weighed was the mesonephros (at day 40 only) thymus, adrenal and pituitary (at day 90 only). The pituitary was not a distinct entity which could easily be dissected at day 40 and the mesonephros was no longer a distinct tissue at day 90. Foetal blood samples were recovered by cardiac puncture, centrifuged at 4°C and the plasmas stored at -15°C until assayed for FSH and inhibin (Smith *et al.*, 1993). The left ovaries were fixed in Bouins fixative and serially sectioned at 4 μm for morphometric studies. The right ovaries were utilized for studies not included in this paper.

The pituitary glands were homogenized into 2 ml of buffer (5 mmol potassium phosphate l^{-1} + 0.1% BSA (w/v) + 1 mmol phenyl methyl sulphonyl fluoride l^{-1}, pH 7.4) as previously described (Smith *et al.*, 1993). The resulting supernatants were stored at -20°C until assayed for FSH.

Morphometric studies

The germ cell number was estimated by the dissector method (Sterio, 1984) and the procedures used are described in detail elsewhere (Smith *et al.*, 1993). During the estimation of number of germ cells, the cells were classified into oogonia, oocytes in meiotic prophase but not surrounded by follicular cells (oocytes), primordial follicles (i.e. an oocyte surrounded by a flattened layer of granulosa cells). Although not observed but looked for were follicles larger than primordial and these were defined as primary follicles (i.e. an oocyte with 1-2 layers of cuboidal granulosa cells) and larger structures were defined as secondary and antral follicles.

RESULTS

Effect of Booroola genotype on foetal characteristics

At day 40 of gestation, the geometric means (and 95% confidence limits) for litter size for the BB and ++ foetuses were 2.4 (2.0, 2.8) and 2.4 (2.2, 2.6) respectively. The effect of Booroola genotype at day 40 is summarized in Table 1. The mean body weight and mean weight of the mesonephros were both significantly lighter (P<0.01) in the BB compared to the ++ genotype. Likewise the mean number of germ cells was significantly lower (P<0.01) in the BB compared to the ++ genotype; at day 40 all the germ cells were present as oogonia. No gene-specific differences in organ weights were noted for the adrenal, thymus (data not shown) or ovary (Table 1).

TABLE 1: Effect of genotype on body weight, ovarian weight, mesonephros weight and number of germ cells of ++ and BB Booroola foetuses at day 40 of gestation. Values are geometric means (and 95% confidence limits)

Foetal characteristic	Booroola genotype* ++	BB
Body weight (g)	4.49[a] (4.26, 4.76)	3.87[c] (3.70, 4.04)
Ovarian weight (mg)	1.16[a] (1.16, 1.39)	1.33[a] (1.17, 1.49)
Mesonephros weight (mg)	16.9[a] (15.6, 18.1)	13.4[c] (12.7, 14.1)
Number of germ cells	49090[a] (37665, 66004)	30905[c] (26942, 35119)
Number of foetuses	10	18

The weights of the ovaries and number of germ cells refer to those of the left ovary. The weights of the mesonephros refer to that on the left side.

*For each row values with different alphabetical superscripts are significantly different. a v c = P<0.01.

At day 90 of gestation, the geometric means (and 95% confidence limits) for litter size for the BB and ++ embryos were 2.7 (2.4, 2.9) and 2.4 (2.0, 2.9) respectively. The germ cells were present as oogonia, oocytes or as primordial follicles. None were present as primary or larger growing follicles. The mean ovarian weight, the total number of germ cells, number of oogonia and number of oocytes were all significantly higher in the BB compared to the ++ genotypes (Table 2). In contrast, the mean body weights (Table 2), adrenal, pituitary and thymus weights (data not shown) and number of primordial follicles (Table 2) were not significantly different between the genotypes. In the ewe, the mesonephros is no longer a distinct entity at day 90 thus no data for this structure were recorded.

Effect of the Inverdale genotype on foetal characteristics

At day 40 nine female foetuses with either I+ or II genotype were obtained from the matings of I rams with I+ ewes but it was not possible to distinguish these two genotypes by body weight or crown-rump length measurements. However 4 of the 9 ovaries had weights that were not different from the authentic I+ ovaries or control ovaries whereas the remaining 5 ovaries were significantly lighter than either the authentic I+ or ++ control ovaries. These foetuses with lighter ovaries were tentatively designated as II (estimated II) whereas the other 4 were tentatively designated as I+ (estimated I+). With this classification system no genotype differences were noted for body weight or mesonephros weight (Table 3) or for thymus or adrenal weight (data not shown). At the time of preparation of this

TABLE 2: Effect of genotype on body weight, gonad weight and germ cell or follicle numbers in ++ and BB Booroola foetuses at day 90 of gestation. Values are geometric means (and 95% confidence limits)

Foetal characteristic	Booroola genotype* ++	BB
Body weight (g)	480[a] (446, 517)	444[a] (407, 485)
Ovarian weight (mg)	14.1[a] (12.4, 15.8)	18.2[b] (15.4, 20.9)
Number of germ cells	290395[a] (223532, 377262)	525444[c] (417148, 661855)
Number of oogonia	76420[a] (51102, 114279)	174730[c] (133922, 227972)
Number of oocytes	161781[a] (111626, 234470)	304674[b] (210762, 440431)
Number of follicles	26003[a] (16413, 41194)	30393[a] (19185, 48149)
Number of foetuses	14	14

The ovarian data are those for the left ovary.
*For each row values with different alphabetical superscripts are significantly different. a v b = P<0.05; a v c = P<0.01 (Student's t-test).

TABLE 3: Effect of genotype on body weight, ovarian weight and mesonephros weight in ++, authentic I+, estimated I+ and estimated II Inverdale foetuses at day 40 of gestation. Values are geometric means (and 95% confidence limits)

Foetal characteristics	Inverdale genotype* ++	I+	Estimated I+	Estimated II
Body weight (g)	4.27[a] (3.93, 4.63)	4.99[a] (4.56, 5.45)	4.31[a] (3.81, 4.86)	4.57[a] (3.84, 5.43)
Ovarian weight (mg)	1.30[a] (1.0, 1.6)	1.4[a] (1.2, 1.7)	1.4[a] (1.2, 1.6)	0.9[b] (0.7, 1.0)
Mesonephros weight (mg)	16.0[a] (14.0, 18.8)	17.0[a] (15.0, 19.0)	14.0[a] (11.0, 17.0)	16.0[a] (13.0, 19.0)
Number of foetuses	15	10	4	5

*For each row values with different alphabetical superscripts are significantly different.
a v b = P<0.05 (Duncan's multiple range test).

paper, the number of germ cells for each of the genotypes at day 40 had not been determined.

The geometric means (and 95% confidence limits) for litter size for the estimated II, estimated I+, authentic I+ and ++ embryos at day 40 were 2.5 (1.7, 3.5), 2.8 (2.2, 3.4), 1.5 (1.2, 1.9) and 1.4 (1.1, 1.7) respectively. The litter sizes for the estimated II and I+ groups were not different from one another but both were different (P<0.05) from the ++ and I+ groups.

At day 90 of gestation the germ cells were present as either oogonia, oocytes or primordial follicles and there were no obvious differences between the body weights or ovarian weights of the ++, I+ or II/I+ genotypes (Table 4). Moreover no differences were noted for the weights of the adrenal, thymus or pituitary (data not shown). When the total populations of germ cells in the ++, authentic I+ and putative II or I+ genotypes were counted 9 of the 15 putative II or I+ ovaries contained similar numbers of germ cells to those found in authentic I+ animals. These foetuses were tentatively designated as I+ animals (estimated I+) (Table 4) whereas the remaining 6 ovaries had germ cell populations which were different to those in the authentic I+ but similar to those in the ++ genotype and were thus designated as II (estimated II) (Table 4). The estimated I+ were also found to have similar numbers of oogonia, oocytes and follicles compared to the authentic I+; these numbers of oogonia and oocytes for both the estimated I+ and authentic I+ were both significantly different (P<0.05) from the estimated II (Table 4).

The geometric means (and 95% confidence limits) for the litter sizes for the estimated II, estimated I+, authentic I+

TABLE 4: Effect of genotype on body weight, ovarian weight and characteristics of germ cells in ++, I+, estimated I+ and estimated II Inverdale foetuses at day 90 of gestation. Values are geometric means (and 95% confidence limits)

Foetal characteristics	Inverdale genotype* ++	I+	Estimated I+	Estimated II
Body weight (g)	513[a] (478, 551)	559[a] (519, 601)	522[a] (468, 583)	545[a] (330, 902)
Ovarian weight (mg)	14.7[a] (5.6,20.4)	14.9[a] (12.4, 17.4)	16.0[a] (9.0, 22.8)	13.3[a] (4.0, 22.7)
Number of germ cells	150090[a] (106924, 210684)	234052[b] (162170, 337795)	243287[b] (166908, 354619)	93451[a] (62254, 140283)
Number of oogonia	29369[a] (17766, 48548)	28029[a] (12756, 61584)	23576[a] (15459, 35594)	1571[b] (33, 74347)
Number of oocytes	73791[a] (46378, 117406)	141492[b] (81846, 224605)	182699[b] (116828, 285710)	61267[a] (38814, 96707)
Number of follicles	32892[a] (26104, 41444)	29732[a] (16625, 53174)	21590[a] (9677, 48169)	24538[a] (19073, 31569)
Number of foetuses	14	10	9	6

The ovarian data are those for the left ovary.
*For each row, values with different alphabetic superscripts are significantly different.
av b = P<0.05 (Duncan's multiple range test).

and ++ groups at day 90 were 2.6 (1.6, 3.8), 2.8 (2.4, 3.1), 1.5 (1.1, 1.9) and 1.0 (1.0, 1.0) respectively. The mean litter sizes for the estimated II and I+ groups were both different ($P<0.05$) from the authentic I+ and ++ groups.

Hormone concentrations in Booroola and Inverdale foetuses at day 90 of gestation

No gene-specific differences were noted for FSH or inhibin in the Booroola or Inverdale foetuses at day 90 (Table 5). Likewise for the pituitary concentrations/contents, no differences for FSH were noted for either the Booroola or Inverdale foetuses (data not shown).

TABLE 5: Plasma concentrations of follicle-stimulating hormone and inhibin in Booroola or Inverdale foetuses at day 90 of gestation. Values are geometric means (and 95% confidence limits)

Booroola or Inverdale genotype	Plasma hormone concentration FSH (ng/ml)	Inhibin (IU/ml)
Booroola		
BB	2.1 (1.5, 2.9)	21.9 (17.5, 27.3)
++	2.3 (1.5, 3.3)	17.5 (12.0, 25.4)
Inverdale		
++	3.0 (2.5, 3.7)	29.6 (23.3, 37.5)
I+	3.0 (2.0, 4.5)	22.7 (17.8, 28.8)
Estimated I+	3.3 (1.4, 6.9)	20.9 (14.1, 30.7)
Estimated II	2.3 (0.7, 5.4)	17.1 (10.0, 28.7)

For the Booroolas and Inverdales separately there were no gene specific differences for either FSH or inhibin.

DISCUSSION

The results of this study show that the Fec^B gene influences body weight, the weight of the mesonephros and the number of germ cells at day 40 of gestation. They also show at day 90 that the Fec^B gene influences ovarian weight as well as the number and maturation of germ cells. Of importance is the finding that the effects of the Fec^B gene are independent of litter size. In early gestation, the mesonephros is a major organ in the developing sheep foetus; at day 40 the mesonephros is ~10 times heavier than the ovary and constitutes ~3% of the total body weight. Previous studies have shown that the mesonephros has a major influence on the ovarian cellular composition, the timing of germ cell maturation and also follicle formation (Zamboni *et al.*, 1979; Byskov, 1986). These observations raise the possibility that the effects of the Fec^B gene on ovarian development may be a consequence of some factor(s) affecting body weight and/or the mesonephros. At day 40 germ cells are in their mitotic proliferative phase, with more germ cells present in the ++ compared to the BB genotype. Our previous studies have shown that at day 75 the number of germ cells has increased to ~$1x10^6$ in both genotypes and that a substantial number have entered into meiosis (Smith *et al.*, 1993). At day 90 the present study shows that the number of germ cells in the ++ genotype had fallen to ~290,000 and in the BB genotype to ~525,000 (Table 2). Collectively these findings for germ cells at days 40 and 90 are consistent with the hypothesis (see Smith *et al.*, 1993) that the times of onset of germ cell mitosis and atresia are retarded in the BB relative to the ++ genotype.

In contrast to the results for Booroola foetuses, those for the Inverdale suggest that there are no effects of genotype on body weight or weight of the mesonephros at day 40. It is unlikely that differences in the litter sizes between the estimated II and I+ groups would have influenced these results as the litter sizes in the authentic I+ and ++ groups were not different and no differences between these two groups were noted for body weight or weight of mesonephros.

The segregation of the female progeny from the I+ ewes mated to I rams at 40 days of gestation into II or the I+ genotype according to ovarian weight was made on the basis that one of the two populations of foetuses had similar ovarian weights to the authentic I+ animals whereas the other population was significantly lighter. If this finding can be confirmed using other criteria it suggests that two but not one copy of the Inverdale gene at day 40 is affecting the ovarian composition without any concomitant differences in the associated mesonephric tissues.

At day 90 of gestation no obvious differences in Inverdale foetuses were noted in ovarian weight between the I+ and ++ groups and, moreover the ovaries in the female progeny from the I+ ewes mated to I rams at day 90 were not separable into two populations by weight. The only obvious differences noted were that the germ cell populations in the II/I+ foetuses were separable into two populations with one population similar to that in the authentic I+. From this we have inferred that the other population was probably from the II foetuses (i.e. est. II). Using this criterion the number of germ cells in the ++ and estimated II groups were not different but both groups had significantly lower mean numbers relative to the authentic and estimated I+ groups. Further studies on the germ cell populations of II, I+ and ++ ewes at day 40 are needed to determine whether the pattern of germ cell maturation for the I+ and II genotypes or similar to different to that for Booroola ewes.

At day 40 of gestation, FSH is not detectable in the heads of foetal sheep or in peripheral plasma (Smith *et al.*, 1993). Likewise in Booroola ewes immunoreactive inhibin is not detectable in ovarian tissue at day 40. In the present study these hormones were not examined in the day 40 foetuses, but given that FSH is not measurable in plasma until day 55 and that inhibin is not measurable in the mesonephros at days 40 or 55 or in the ovary until day 75 (Smith *et al.*, 1993) it is unlikely that these hormones are associated in any way with the genotypic differences observed in the ovaries or mesonephros at day 40 of gestation. Moreover the lack of any gene-specific differences in plasma or pituitary FSH or plasma inhibin in either the Booroola or Inverdale genotypes at day 90 suggest that the changes taking place in the ovaries at that time are independent of both of these hormones.

In summary the findings that there were differences in body weight and weight of the mesonephros in Booroola but

not Inverdale foetuses at day 40 suggest that the mechanisms leading to the genotypic differences in foetal Booroolas and Inverdales are not the same. Nevertheless the results are consistent with the hypothesis that the Fec^B and Fec^I genes influence ovarian development in foetal life.

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